A Nonfeminizing Estrogen Analog Protects against Ethanol Withdrawal Toxicity in Immortalized Hippocampal Cells

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ABSTRACT

We have shown that 17β-estradiol protects against ethanol withdrawal toxicity in rats. Here, we investigated whether a cellular model of ethanol withdrawal could be developed in a cultured hippocampal cell line (HT22) and whether an adamantyl-containing nonfeminizing estrogen analog, ZYC26 [(3-hydroxy-2-adamantyl(1)-4-methyl-estra-1,3,5(10)-17-one), protects against ethanol withdrawal toxicity. HT22 cells were exposed to ethanol (0–500 mM) for 24 h in the presence or absence of ZYC26 or 17β-estradiol. The ethanol solution was then removed from the cells for 4 h to create ethanol withdrawal. Samples were collected at the end of a 24-h ethanol exposure or at 4 h of ethanol withdrawal to assess cell viability using a calcein assay, lipid peroxidation by measuring malondialdehyde, and protein oxidation by measuring carbonyl contents. When tested, ethanol concentrations were constantly maintained during a 24-h ethanol exposure and eliminated at 4 h of ethanol withdrawal. Ethanol withdrawal decreased cell viability and increased the levels of malondialdehyde and carbonyls more than ethanol exposure. ZYC26 reduced the cell death and malondialdehyde levels at a lower dose (1 μM) than 17β-estradiol (10 μM). The increased carbonyl contents were reduced only by ZYC26 treatment. These data suggest that ethanol withdrawal can be created in HT22 cells in a manner that is more toxic than ethanol exposure and that ZYC26 is a more potent cytoprotectant than 17β-estradiol against cell death and oxidative damage induced by ethanol withdrawal. Therefore, ZYC26 can be a potential alternative estrogen therapy for a cellular and oxidative imbalance associated with ethanol withdrawal.

Estrogen analogs are referred to as synthetic or natural chemical compounds that resemble the major endogenous estrogen 17β-estradiol in structure and/or function. In recent years, certain estrogen analogs have been made that exert an increased neuroprotective activity and a decreased feminizing activity (Simpkins et al., 2004). Such properties of estrogen analogs can be a great advantage over 17β-estradiol considering unwanted side effects of 17β-estradiol on reproductive organs. In the current study, using an adamantyl-estrogen analog without feminizing effects (Simpkins et al., 2004), we investigated whether the estrogen analog protects against ethanol withdrawal-induced cytotoxicity and oxidative insults.

We have reported previously that 17β-estradiol protects against ethanol withdrawal insults at the neuronal, behavioral, and a signal transduction level in rats (Jung et al., 2005). In terms of oxidative stress associated with ethanol withdrawal, experimental animals and alcohol-dependent individuals showed increased oxidative markers of malondialdehyde or free hydroxyl radicals during ethanol withdrawal (Marotta et al., 1997; Vallett et al., 1997; Jung et al., 2005). In particular, brain tissues obtained from ethanol-withdrawn female rats showed enhanced lipid peroxidation in a manner that correlates with behavioral impairment and that is prevented by 17β-estradiol treatment (Jung et al., 2004). Although such in vivo model systems have provided important evidence regarding the neuronal and behavioral consequences of ethanol withdrawal, an in vitro system would allow more focused mechanistic assessment. An in vitro model of ethanol withdrawal has been used in other laboratories in which ethanol withdrawal induced the excitatory synaptic responses in cultured hippocampal cells (Thomas et al., 1998). We used an immortalized hippocampal cell line, HT22, because this cell line has been used to demonstrate protective effects of nonfeminizing estrogens. For instance, a structural analog of 17β-estradiol (enantiomer of 17β-estradiol) protected against HT22 cell death induced by oxidative stress (Green et al., 2001). Another nonfeminizing estrogen

ABBREVIATIONS: ZYC26, (3-hydroxy-2-adamantyl(1)-4-methyl-estra-1,3,5(10)-17-one; AM, acetoxymethylester; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
analog, 2-adamantyl-estrone, protected against glutamate-induced cytotoxicity in HT22 cells (Liu et al., 2002). The protective effects of estrogen analogs were also shown in other cell line such that 17α-estradiol, an isomer of 17β-estradiol, protected against cell loss induced by serum deprivation in human neuroblastoma cells (Green et al., 1997). Importantly, these estrogen analogs interact only weakly or not at all with known estrogen receptors (Perez et al., 2006), suggesting that neuroprotection induced by these analogs does not necessarily require estrogen receptors.

ZYC26, used in this study, contains four rings with phenolic A ring. The phenolic hydroxyl group of estratrienes’ A ring donates a hydrogen radical that can subsequently quench free radicals formed in oxidative stress conditions (Prokai and Forster, 2004; Dykens et al., 2005; Perez et al., 2006). ZYC26 also contains an adamantyl group at the C2 position of estrone. This bulky electron-donating substituent provides two advantages over 17β-estradiol: it decreases the affinity of the compound for estrogen receptors that mediate feminizing side effects (Perez et al., 2006) and enhances the stability of the phenolic radical that plays an important role in antioxidant activity (Xia et al., 2002). Furthermore, structure-activity relationship evaluations (Green et al., 1997; Perez et al., 2006) indicate that estrogenicity bears no relationship to neuroprotection among estrogens. As a result, such compounds may provide enhanced neuroprotection and antioxidant activity at doses similar to those of natural estrogens without feminizing side effects (Liu et al., 2002).

In this study, we assessed the ability of ZYC26 to protect against the adverse effects of ethanol withdrawal on cell viability and oxidative damage using an in vitro model of ethanol withdrawal in the HT22 cell. Cell viability was assessed using a calcine assay in which a fluorogenic esterase permeates only live cells. Oxidative damage was assessed by measuring products of lipid peroxidation malondialdehyde and by measuring the carbonyl contents of proteins. The protective effects of ZYC26 against ethanol withdrawal toxicity were compared with those of 17β-estradiol.

Materials and Methods

Reagents. ZYC26 was made in our laboratories using methods described previously (Liu et al., 2002; Perez et al., 2006). 17β-Estradiol was purchased from Steraloids (Wilton, NH). HT22 cells, a murine hippocampal cell line, were the generous gift of Dr. David Schubert (Salk Institute, San Diego, CA).

Cell Culture. HT22 cells were maintained and treated as described previously (Perez et al., 2005). Cell viability was determined using the calcein-acetoxyxymethylester (AM) assay (Perez et al., 2005). The HT22 line was originally selected from HT4 cells that were immortalized from primary hippocampal neurons using a temperature-sensitive small virus-40 T antigen (Morimoto and Koshubert, 2005). The HT22 line was originally selected from HT4 cells that were immortalized from primary hippocampal neurons using a temperature-sensitive small virus-40 T antigen (Morimoto and Koshubert, 2005). The HT22 line was originally selected from HT4 cells that were immortalized from primary hippocampal neurons using a temperature-sensitive small virus-40 T antigen (Morimoto and Koshubert, 2005). The HT22 line was originally selected from HT4 cells that were immortalized from primary hippocampal neurons using a temperature-sensitive small virus-40 T antigen (Morimoto and Koshubert, 2005).

Cell viability was determined using the membrane-permeant calcein-AM dye (Molecular Probes, Eugene, OR). Cell viability was determined using the calcein-acetoxyxymethylester (AM) assay (Perez et al., 2005). The HT22 line was originally selected from HT4 cells that were immortalized from primary hippocampal neurons using a temperature-sensitive small virus-40 T antigen (Morimoto and Koshubert, 1990). HT22 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and gentamicin (50 μg/ml), at 37°C in an atmosphere containing 5% CO2 and 95% air. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin at concentrations ranging from 0.063 to 1 mg/ml as a standard curve.

Experimental Treatments. For the assessment of cell viability, HT22 cells were plated into 96-well tissue culture plates at 4000 cells per well in 100 μl of cell culture medium. For the assessment of malondialdehyde and carbonyl contents, HT22 cells were plated into Petri dishes (Greiner Bio-One, Monroe, NC) at approximately 1 × 10⁶ cells per dish to extract sufficient amounts of protein for the assays. The following day, the cells were continuously exposed to 0, 50, 100, 200, or 500 mM ethanol for 24 h. The culture plates or Petri dishes were tightly sealed with parafilm immediately after ethanol treatment to prevent ethanol evaporation. For the continuous ethanol exposure condition, cell viability and oxidative markers were measured at the end of the 24-h ethanol exposure. In withdrawal experiments, the ethanol-containing medium was replaced by vehicle-containing medium for 4 h after the 24-h ethanol exposure (Li and Kendig, 2003; Mostalino et al., 2004). At the end of ethanol withdrawal, cell viability and oxidative markers were measured. ZYC26 and 17β-estradiol stock solutions were prepared at a concentration of 1 and 10 μM in dimethyl sulfoxide and were given to cells during the 24-h ethanol exposure. Vehicle-treated control cultures were exposed in parallel to the same concentration of dimethyl sulfoxide present in the experimental cultures. There were no measurable effects of dimethyl sulfoxide vehicle treatment on cell viability under these conditions.

Ethanol Concentrations in the HT22 Cell Culture. To determine ethanol concentrations in HT22 cells, the cells were treated with vehicle or 50, 100, or 200 mM ethanol. Ten microliters of sample solution was collected at six different time points: 0 (immediately after ethanol treatment), 2, 4, 6, and 24 h after ethanol treatment and 4 h after the ethanol solution was replaced with a vehicle-containing media. The 10 μl of sample solution was added to 200 μl of ice-cold 0.55 M perchloric acid. The media were neutralized with 200 μl of 0.6 M KOH containing 50 mM acetic acid. This solution precipitates the perchlorate ion and buffers the solution to approximately pH 5. After the samples were centrifuged, the resulting supernatant was used to measure ethanol concentration using an enzymatic assay (Smolen et al., 1986). A 20-μl aliquot of the supernatant solution was pipetted into duplicate assay tubes and one blank tube. The assay solution consisted of 2.29 mM NAD (Roche Diagnostics, Indianapolis, IN), 30 U of yeast alcohol dehydrogenase (Sigma-Aldrich, St. Louis, MO), and 500 mM Tris-HCI, pH 8.8, in a total volume of 140 μl. The blank tube contained no alcohol dehydrogenase. The reaction mixture was allowed to incubate at room temperature for 30 min before measuring the absorbance of nicotinamide adenine dinucleotide formed at 340 nm in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Ethanol concentrations were calculated from linear regression analysis of a standard curve of known ethanol concentrations.

Calcine-Acetoxyxymethylester Viability Assay. Cell viability was quantitated using the membrane-permeant calcine-AM dye (Molecular Probes, Eugene, OR). Calcine-AM is a fluorogenic esterase substrate that easily permeates live cells that have esterase activity and membranes. Once hydrolysis of calcine-AM by intracellular esterases begins, it produces calcine, a strongly fluorescent compound that is well retained in the cell cytoplasm, which enables us to measure relative fluorescent units. After the removal of the medium from the 96-well plates, the cells were rinsed once with phosphate-buffered saline (PBS; pH 7.4) and incubated in a solution of 2.5 μM calcine-AM in PBS. Twenty minutes later, fluorescence was determined using a Bio-Tek FL600 microplate reader (Bio-Tek Instruments, Winooski, VT) with an excitation/emission filter set at 485/530 nm. Cell culture wells treated with methanol served as blanks. The results, obtained in relative fluorescent units, are expressed as the percentage of vehicle-treated control values. When using this protocol, there is a linear relationship between the number of viable cells per well and measured calcine-AM fluorescence when viable cell numbers were between 300 and 5000 cells per well (r² = 0.9991). For imaging of cell viability during ethanol withdrawal, separate HT22 cells were treated with vehicle or ethanol (200 μM) as described above. Cell culture plates were then placed on the confocal microscope platform at 4 h of ethanol withdrawal and scanned with a laser set at the wavelength (485/530 nm) appropriate for the calcine-AM. HT22 cell death induced by excitotoxin is consistent with previous reports using alternative viability methods, such as trypan blue dye exclusion.
whether ZYC26 was more potent than 17β-estradiol for alcohol withdrawal were different from ethanol exposure and ahoc test was also conducted to determine whether the effects of ethanol withdrawal condition without hormone treatment. The post hoc groups were different from an ethanol-free condition or an isons test (post hoc test) was conducted to determine which treat-ance (ANOVA) or two-way ANOVA with a Tukey’s multiple-compar- between groups was detected (\( p < 0.05 \)).

Malondialdehyde Assay. The levels of malondialdehyde were assessed using a colorimetric assay (Calbiochem, San Diego, CA) in which a chromogenic reagent reacts with malondialdehyde and yields a stable chromophore with maximal absorbance at 586 nm. Cells treated with ethanol were washed with PBS, centrifuged (100,000 \( \times g \) for 60 min at 4°C), and suspended in 1 ml of PBS. Cell lysis was then done by sonication in the presence of butylated hydroxytoluene (0.5 M) to prevent sample oxidation during preparation. Cell homogenates were added to a chromogenic reagent and incubated at 45°C for 60 min. After the samples were cooled off, absorbance was read at 586 nm.

Protein Carbonyl Assay. Carbonyl concentrations were measured according to the method of Levine et al. (1990). Cells treated with ethanol were centrifuged at 200g for 5 min, 100 \( \mu l \) of 2 N HCl was added, and then they were incubated for 1 h at room temperature in the dark. Trichloroacetic acid (20%) was added, and then the cells were incubated on ice for 10 min and centrifuged for 5 min at 5000 rpm. The pellets were washed, sonicated, and centrifuged for 10 min at 5000 rpm. The pellets were resuspended in buffer, dissolved overnight, and read at 360 nm. The results were expressed as nanomoles carbonyl per milligram of protein.

Correlation Assay. Separate HT22 cells were exposed to vehicle or ethanol (100 and 200 mM) for 24 h and went through withdrawal for 4 h. After 4 h of ethanol withdrawal, the same cell cultures were used to assess cell viability, malondialdehyde levels, and carbonyl contents, as described above. Pearson correlation coefficients (Hines et al., 2000; Kuo et al., 2003) were calculated to determine whether samples with lower cell viability have higher levels of malondialde- hyde or carbonyls.

Statistical Analysis. Each dose/time group contained seven to 10 observations, and each experiment was repeated three to five times. Data are presented as mean \( \pm \) S.E.M. The significance level for all data analysis was set at \( p < 0.05 \). The following statistical methods were applied.

Repeated measures analysis by a factor of ethanol dose was conducted for all data analysis of cell survivals or ZYC26 effects between different treatment groups. When an overall significant difference between groups was detected \( (p < 0.05) \), one-way analysis of variance (ANOVA) or two-way ANOVA with a Tukey’s multiple-comparisons test (post hoc test) was conducted to determine which treatment groups were different from an ethanol-free condition or an ethanol withdrawal condition without hormone treatment. The post hoc test was also conducted to determine whether the effects of ethanol withdrawal were different from ethanol exposure and whether ZYC26 was more potent than 17β-estradiol.

A one-way ANOVA was conducted to determine whether there was a difference in malondialdehyde levels or protein carbonyl among groups [a vehicle group, three ethanol exposure groups (50, 100, and 200 mM), and three ethanol withdrawal groups (50, 100, and 200 mM)] followed by Tukey’s post hoc test. Pearson correlation coefficients (Hines et al., 2000; Kuo et al., 2003) were calculated to assess the association between the magnitude of cell death and the levels of malondialdehyde or protein carbonyls. Pearson correlation coefficient was suitable in this case because both \( x \) (malondialdehyde or protein carbonyls) and \( y \) (cell viability) variables were dependent variables. Nonlinear regression analysis was conducted to calculate \( ED_{50} \) of ZYC26 or 17β-estradiol.

Results

Ethanol Concentrations in the HT22 Cells. We first assessed the ethanol concentrations in the HT22 cells as a function of time after ethanol treatment. When measured immediately after ethanol treatment (0 h), 50, 100, and 200 mM ethanol treatment yielded 2.3, 4.6, and 9.4 mg/ml ethanol concentrations in the cells, respectively. At 24 h of etha-nol exposure, 50, 100, and 200 mM ethanol treatment yielded 2.15, 3.98, and 8.5 mg/ml ethanol concentrations in the cells, respectively. These data indicate that ethanol concentrations did not significantly change over the 24-h ethanol exposure under the current experimental condition. In contrast, 4 h after the ethanol-containing media were replaced with vehicle media (at 4 h of ethanol withdrawal), no ethanol was detected in the cultures (data not shown).

The Effects of Ethanol Withdrawal on Cell Viability. The effects of ethanol withdrawal on cell viability were assessed as a function of ethanol exposure duration (hours). HT22 cells were exposed to 4, 8, 12, 24, or 48 h of ethanol at 0 (vehicle), 50, 100, or 200 mM. The cells were then withdrawn for 4 h, and cell viability was measured at the end of the 4-h ethanol withdrawal. All ethanol-treated cells showed a significantly decreased viability compared with ethanol-free control cells (dash line at 100%) in a manner that depended on concentrations of ethanol \( [\mathcal{F}(3,205) = 356, p < 0.001] \) and duration of ethanol exposure \( [\mathcal{F}(5,205) = 296, p < 0.001] \) (Fig. 1). These data suggest that a longer exposure and a higher concentration of ethanol produce a greater ethanol withdrawal stimulus, which results in a greater cell death.

Fig. 1. The effects of ethanol withdrawal on cell viability. HT22 cells were exposed to vehicle or ethanol (100, 200, or 500 mM) for 4, 8, 12, 24, or 48 h and withdrawn for 4 h. Using calcein assay, cell viability was measured at the end of the 4-h ethanol withdrawal. To compare the cell death during ethanol withdrawal and ethanol exposure, cell viability was also measured at the end of a 24-h ethanol exposure whereas cells were under ethanol influence. All ethanol withdrawal cells showed a significantly decreased viability compared with ethanol-free control cells (dash line at 100%) \( (p < 0.001) \). For the comparison with ethanol exposure, ethanol withdrawal resulted in a greater cell death than ethanol exposure \( (*, p < 0.01\) at 100 and 200 mM ethanol). The x-axis indicates ethanol concentrations (millimolar). Depicted are mean \( \pm \) S.E.M. for \( n = 8 \).
To compare the cell death during ethanol withdrawal and ethanol exposure, we also measured cell viability at the end of ethanol exposure for 24 h, whereas cells were under ethanol influence. We found that, although ethanol exposure suppressed cell viability, more cell death occurred during ethanol withdrawal at 100 and 200 mM ethanol (p < 0.001 at both doses, by Tukey’s post hoc test). There was no difference in cell viability between ethanol withdrawal and ethanol exposure at the highest concentration (200 mM) of ethanol tested.

Control Experiment. Our results of the converse experiment demonstrated that the cell death during ethanol withdrawal was not due to cell removal that might have occurred, whereas ethanol-containing media were replaced by vehicle media. Cultures that were exposed to and replaced with vehicle media maintained 100% cell viability at 24 h of exposure to media and 4 h after the media change. Compared with these cells, when vehicle media were replaced with ethanol media (100 or 200 mM) for 4 h, lower cell viability was observed [F(2,79) = 13, p < 0.001 by dose; F(2,79) = 18, p < 0.001 by time points]. These data demonstrate that cells were not removed by the media change (Fig. 2).

The Effect of Ethanol Withdrawal on Malondialdehyde Levels. We then assessed whether HT22 cells exposed to ethanol for 24 h and withdrawn for 4 h produce lipid peroxidation by measuring malondialdehyde levels. Both ethanol exposure and ethanol withdrawal significantly increased malondialdehyde levels at 100 (p < 0.05) and 200 mM (p < 0.01) ethanol compared with ethanol-free control cells [F(6,44) = 16, p < 0.001, n = 7]. The increases in the malondialdehyde levels were more prominent during ethanol withdrawal than during ethanol exposure at 100 (p < 0.01) and 200 mM (p < 0.05) ethanol (Fig. 3). These results demonstrate that ethanol withdrawal produces lipid peroxidation in HT22 cells to a greater degree than ethanol exposure per se.

The Effect of Ethanol Withdrawal on Protein Carboxyls. When oxidative damage to protein was assessed by measuring carbonyl contents, both ethanol exposure and ethanol withdrawal increased carbonyl contents compared with ethanol-free control cells [F(6,46) = 62, p < 0.001, n = 7]. The increases in the carbonyl contents were more prominent during ethanol withdrawal than during ethanol exposure at 100 (p < 0.05) and 200 mM (p < 0.01) ethanol (Fig. 4). These results demonstrate that ethanol withdrawal produces greater protein oxidation than ethanol exposure per se in this cell line.

Correlation between Cell Viability and Malondialdehyde or Protein Carboxyls. Pearson correlation coefficients were calculated to assess the relationship between cell death and oxidative markers of lipid peroxidation or protein oxidation induced by ethanol withdrawal. During ethanol withdrawal, we found a positive correlation between cell viability and malondialdehyde levels (r = 0.62, p < 0.001) and a positive correlation between cell viability and protein carboxyl contents (r = 0.68, p < 0.001). These data suggest that ethanol withdrawal produces greater oxidative damage to protein than ethanol exposure per se in this cell line.
withdrawal, there was an association between the magnitude of cell death and the levels of malondialdehyde or protein carbonyls such that cell treatments showing higher cell death had higher levels of malondialdehyde (Pearson coefficient $r^2 = -0.76, p < 0.01$) or protein carbonyls (Pearson coefficient $r^2 = -0.85, p < 0.01$) (data not shown). These data suggest that cell death induced by ethanol withdrawal was related to oxidative damage in HT22 cells.

**The Effect of ZYC26 on Ethanol Withdrawal-Induced Cytotoxicity.** To determine whether an estrogen analog (ZYC26) protects against ethanol withdrawal toxicity in this cell line, we treated cells with ZYC26 (0, 1, and 10 μM, $p < 0.01$) or 17β-estradiol (0, 1, and 10 μM) at the same time cells were treated with ethanol for 24 h (Fig. 5A). When assessed 4 h after ethanol was removed from cells [F(5,140) = 154, $p < 0.001$], cells treated with ZYC26 showed higher survival compared with vehicle-treated ethanol-withdrawn cells ($p < 0.01$ at both 1 and 10 μM ZYC26) at all doses of ethanol. In comparison, 17β-estradiol treatment decreased cell death induced by ethanol withdrawal only at its high dose (10 μM, $p < 0.01$) and at certain doses of ethanol (100 and 200 mM). At the same dose (10 μM) of these estrogens, cell protection was much greater with ZYC26 than with 17β-estradiol ($p < 0.01$ at 50 and 200 mM ethanol, $p = 0.004$ at 100 mM ethanol). These data demonstrate that ZYC26 more potently protects against cell death induced by ethanol withdrawal than 17β-estradiol.

Microscopic examination of cell viability was conducted with or without ZYC26 (1 μM) at 4 h of ethanol withdrawal for cells exposed to 100 mM ethanol for 24 h (Fig. 5B). Consistent with the results from the calcein assay, a significantly lower cell population was observed during ethanol withdrawal (center) compared with ethanol-free cells (left). ZYC26 (1 μM) treatment (right) partially prevented cell death induced by ethanol withdrawal. For cell viability assessment, we finally measured ED$_{50}$ of these estrogens for a potency comparison (Fig. 5C). ED$_{50}$ of ZYC26 (110 nM) was much lower than ED$_{50}$ of 17β-estradiol (3.4 μM), suggesting that ZYC26 is a more potent neuroprotectant than 17β-estradiol against HT22 cell death induced by ethanol withdrawal [F(1,56) = 144 by estrogen treatment, $p < 0.001$; F(3,56) = 110 by ethanol dose, $p < 0.001$].

**The Effect of ZYC26 on Ethanol Withdrawal-Induced Lipid Peroxidation.** To determine whether ZYC26 protects against ethanol withdrawal-induced lipid peroxidation, we treated cells with ZYC26 (0, 1, and 10 μM, $p < 0.01$) or 17β-estradiol (0, 1, and 10 μM) at the same time cells were treated with ethanol for 24 h (Fig. 6). Cells under ethanol withdrawal insults produced two to six times the levels of malondialdehyde ($p < 0.01$) than ethanol-free cells (100% dash line = 79 mM malondialdehyde/mg), indicating that ethanol withdrawal produces lipid peroxidation. Both ZYC26 and 17β-estradiol treatments decreased the malondialdehyde levels at 100 ($p < 0.05$) and 200 ($p < 0.01$) mM ethanol, in a manner that depended on the dose of 17β-estradiol or ZYC26 ($p < 0.05$). At the same dose (10 μM) of the estrogens, ZYC26 decreased the malondialdehyde levels to a greater degree than 17β-estradiol ($p < 0.01$), suggesting that ZYC26 exerts more potent antioxidant activity than 17β-estradiol [F(4,120) = 11, $p < 0.001$ by estrogen treatment; F(4,120) = 11, $p < 0.001$ by ethanol concentration].

![Image](https://example.com/image.png)
withdrawal-induced protein oxidation. Figure 7 illustrates that cells exposed to and withdrawn from ethanol showed increased protein carbonyl contents in a manner that depended on the concentrations of ethanol compared with ethanol-free cells [100% dash line = 0.94 nM carbonyls/mg proteins, \( F(3,120) = 1537 \) by ethanol concentration, \( p < 0.001 \)]. Compared with vehicle-treated ethanol withdrawal cells, only 10 \( \mu \)M ZYC26 (\( p < 0.001 \)) decreased carbonyl contents at 100 (\( p < 0.001 \)) and 200 mM (\( p < 0.001 \)) ethanol. \( F(4,120) = 106 \) by estrogen treatment, \( p < 0.001 \); \( F(3,120) = 106 \) by ethanol concentration, \( p < 0.001 \).

**Discussion**

In humans and experimental animals, abrupt termination of chronic ethanol consumption damages brain to a greater degree than ethanol exposure itself (Sullivan et al., 2000; Jung et al., 2004). In this study, we demonstrated that an in vitro model for ethanol withdrawal could be developed in an HT22 cell line and that ethanol withdrawal exerted a greater toxicity to these cells than ethanol per se. Furthermore, we demonstrated that 17\( \beta \)-estradiol and a nonfeminizing estrogen analog are able to reduce the pro-oxidant effects of EW in this cell line.

Initially, we tested the cytotoxicity of ethanol withdrawal as a function of ethanol exposure duration. Typically in in vivo situations, the magnitude of ethanol withdrawal signs and symptoms depends on the length and dose of ethanol administration (Lal et al., 1988). We were able to mimic this phenomenon by showing that ethanol withdrawal-induced cell death was greater after a longer ethanol exposure than a shorter ethanol exposure. Critical to the interpretation of the present studies is the maintenance of the intended ethanol concentrations. Ethanol-containing media in HT22 cells showed constant ethanol concentrations for 24 h, whereas no ethanol was detected 4 h after the removal of ethanol media. Such abrupt changes in ethanol levels do not completely model the in vivo kinetics of ethanol with an increase and a gradual decrease in ethanol level (Rewal et al., 2005). Nonetheless, this observation substantiates that cells were under the ethanol influence and were completely withdrawn. Consequently, cell death occurred, which we believe is a manifestation of ethanol withdrawal toxic effects, not an artifact due to cell removal by media change. This was demonstrated in our control experiment where 100% cell viability was maintained in the cells exposed to and replaced with vehicle media.

Our findings indicate that ethanol withdrawal is pro-oxidant, whereas 17\( \beta \)-estradiol and ZYC26 are antioxidants, because products of lipid peroxidation and protein oxidation were enhanced during ethanol withdrawal in a manner ameliorated by 17\( \beta \)-estradiol and ZYC26 treatment. In addition, high correlation coefficients between cell death and the levels of malondialdehyde or protein carbonyls imply that the prooxidant ethanol withdrawal stimulus may produce functional damage. Supportive of this view, ethanol withdrawal-induced spontaneous seizure activity correlated with the production of oxidative hydroxyl radicals in ethanol-withdrawn rats (Vallett et al., 1997). Similarly, the levels of excitatory neurotransmitters correlated with oxidative stress markers in abstinent human alcoholics' cerebrospinal fluid (Tsai et al., 1998). In contrast to the pro-oxidant effects of ethanol withdrawal, estrogen has been recognized for its antioxidant properties. Our findings that the cytoprotective effects of two estrogen compounds were seen at concentrations similar to their antioxidant effects are consistent with others' findings showing a correlation between the neuroprotective effects and antioxidant effects of estrogen (Sagara, 1998; Moosmann and Behl, 1999).

Accumulating evidence suggests that structural analogs of 17\( \beta \)-estradiol are also neuroprotective. Such estrogen ana-
logs interact only weakly or not at all with known estrogen receptors, providing for neuroprotective efficacy (Green et al., 2001; Perez et al., 2006). The neuroprotection afforded by estrogen analogs has been reported in a variety of cell lines and animal models. The enantiomer of 17β-estradiol binds only weakly to either estrogen receptor α or β but is as effective as 17β-estradiol in protection against cell death induced by pro-oxidant H₂O₂ in cultured human lens epithelial cells (Wang et al., 2003), in human SK-N-Sh neuroblastoma cells, and in HT22 cells (Green et al., 2001). In a rodent model of focal ischemia, both 17β-estradiol and enantiomer of 17β-estradiol treatment resulted in a significant reduction in lesion volume (Green et al., 2001). The enantiomer of 17β-estradiol, at the doses effective in that study, did not stimulate uterine growth or vaginal opening in juvenile female rats when administered daily for 3 days (Green et al., 2001). Furthermore, phytoestrogen without feminizing effects conferred protective effects on the cardiovascular system (Dubey et al., 1999). Collectively, these studies suggest that the estrogen analogs are neuroprotective in vitro and in vivo models and can be disassociated from the peripheral estrogenic actions.

The HT22 cells lack ionotropic glutamate receptors such as N-methyl-D-aspartate receptors (Zaulyanov et al., 1999). Upon activation of N-methyl-D-aspartate receptors, Ca²⁺ passes through a channel in the center of the receptor complex, triggering excitatory signals. Therefore, the observed HT22 cell death in the current study is less likely through excitotoxicity involving glutamate receptors. Instead, HT22 cells contain the glutamate/cystine antiporter, which is required for the delivery of cystine into neuronal cells and for the export of glutamate out of cells (Sato et al., 1999). This is a useful feature of this cell line as an oxidative model because cystine is required for the synthesis of an endogenous antioxidant glutathione in neuronal cells (Schubert and Piasecki, 2001). A study reports that excess extracellular glutamate completely inhibits the uptake of cystine (Sagara, 1998) that can deplete intracellular antioxidant glutathione. Glutamate is the primary excitatory neurotransmitter in the brain, and ethanol withdrawal is associated with increased extracellular glutamate levels (Rossetti and Carboni, 1995). Given this information, it is possible that the excitotoxic ethanol withdrawal up-regulates the availability of extracellular glutamate that ultimately counteracts against the endogenous antioxidant system. Previously, HT22 cells were thought to lack functional nuclear estrogen receptors (Behl et al., 1995). However, a recent study reported that HT22 cells contain estrogen receptor α and these receptors mediate 17β-estradiol protection against glutamate toxicity (Deecher et al., 2005). The exact reason for this discrepancy is not clear at this moment. It may be due to multiple factors including culturing conditions or passage number of this cell line. Nonetheless, sufficient evidence suggests that estrogen receptors are not required for the protective effects of estrogen analogs. Using these cells, our laboratory has shown that 2-adamantyl estrone did not bind to either human recombinant estrogen receptor α or estrogen receptor β but protected against glutamate toxicity (Liu et al., 2002). Dhandapani and Brann (2002) also showed that estrogens at pharmacological doses activated a free radical-scavenging pathway independently of estrogen receptors. Furthermore, because estrogen receptors mediate feminizing effects, the failure of ZYC26 treatment to increase uterine weights in female rats (E. Perez and J. W. Simpkins, unpublished data) substantiates that ZYC26 is a nonfeminizing estrogen compound that works independent of estrogen receptors.

ZYC26 contains an adamantyl group at the C2 position of estrone. This structural configuration confers an advantage over 17β-estradiol in terms of antioxidant activity because the bulky electron-donating substituent (the adamantyl group) decreases the affinity of the compound for estrogen receptors (Xia et al., 2002). The donated hydrogen radical can quench free radicals formed in oxidative stress conditions. Furthermore, the adamantyl group enhances the stability of the adjacent phenolic radical, which is an essential element of antioxidant properties (Dhandapani and Brann, 2002). Miller et al. (1996) also reported that 2- and 4-additions to the phenolic A-ring of estradienes are potent antioxidants because the additions stabilize a phenolic group that scavenges free radicals. In fact, estrogen compounds with electron-donating substituents adjacent to the phenolic radical have been reported to be better neuroproteonants than 17β-estradiol (Green et al., 1997; Moosmann and Behl, 1999). Supportive of this view is a study showing that 2-adamantyl estrone protected retinal ganglion cells against glutamate-induced cytotoxicity (Kumar et al., 2005). The greater stability of the phenolic radical seems to provide greater neuroprotective and antioxidant activity, and this could explain why ZYC26 was more potently protective against cell death and oxidative insults than 17β-estradiol in our studies.

We observed that both 17β-estradiol and ZYC26 were more effective in protecting against lipid peroxidation than against protein carbonylation during ethanol withdrawal. This is probably due to the intercalation of lipid-soluble estrogens into membranes, where they cycle redox events (Prokai and Forster, 2004), thereby preventing the propagation of lipid peroxidation (Dykens et al., 2005). In contrast, the majority of protein oxidation occurs in the cytosol, where estrogen concentrations are low.

In conclusion, we found that the HT22 cell model of ethanol withdrawal recapitulates the oxidative insults of ethanol withdrawal that we previously observed in animals and demonstrated that ethanol withdrawal is more toxic than ethanol exposure. The more potent antioxidant effects of ZYC26 than 17β-estradiol in this ethanol withdrawal model suggest that an adamantyl-containing estrogen analog is worthy of further study for a potential alternative estrogen therapy in alcoholism.

References
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